NOVEL HUMAN ION EXCHANGER PROTEINS AND POLYNUCLEOTIDES ENCODING THE SAME

The present application claims the benefit of U.S. Provisional Application Number 60/263,384 which was filed on January 23, 2001 and is herein incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates to the discovery, identification, and characterization of novel human polynucleotides encoding a novel human ion exchanger protein that shares sequence similarity with other mammalian ion exchanger proteins. The invention encompasses the described polynucleotides, host cell expression systems, the encoded proteins, fusion proteins, polypeptides and peptides, antibodies to the encoded proteins and peptides, and genetically engineered animals that either lack or overexpress the disclosed genes, antagonists and agonists of the proteins, and other compounds that modulate the expression or activity of the proteins encoded by the disclosed genes, which can be used for diagnosis, drug screening, clinical trial monitoring, the treatment of diseases and disorders, and cosmetic or nutriceutical applications.

2. BACKGROUND OF THE INVENTION

Membrane proteins can serve as recognition markers, mediate signal transduction, and can mediate or facilitate the passage of materials across the lipid bilayer. As such, membrane proteins, are proven drug targets. Ion exchangers, or ion transport proteins, are expressed in the plasma membrane of animal cells. Sodium/calcium exchangers, for example, extrude calcium in parallel with the plasma membrane ATP-driven calcium pump. As many ion transporters are reversible, sodium/calcium

transporters can also mediate the entry of calcium. Cellular increases in the concentration of sodium lead to increases in the concentration of calcium as mediated by the sodium/calcium exchanger. This activity is important in the action of cardiac therapeutic digitalis. Similarly, alterations in sodium and calcium concentrations can modulate the conductance of some epithelia, signaling in some sense organs (*e.g.*, photoreceptors and olfactory receptors) and calcium-dependent secretion in neurons and secretory cells.

3. SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification, and characterization of nucleotides that encode novel human proteins and the corresponding amino acid sequences of these proteins. The novel human ion exchanger proteins (NHIEPs) described for the first time herein share structural similarity with mammalian sodium-calcium exchanger proteins, and potassium dependent versions of the same.

The novel human nucleic acid sequences described herein encode alternative proteins/open reading frames (ORFs) of 921 and 620 amino acids in length (SEQ ID NOS: 2 and 4).

The invention also encompasses agonists and antagonists of the described NHIEPs, including small molecules, large molecules, mutant NHIEPs, or portions thereof, that compete with native NHIEP, peptides, and antibodies, as well as nucleotide sequences that can be used to inhibit the expression of the described NHIEPs (*e.g.*, antisense and ribozyme molecules, and open reading frame or regulatory sequence replacement constructs) or to enhance the expression of the described NHIEPs (*e.g.*, expression constructs that place the described polynucleotide under the control of a strong promoter system), and transgenic animals that express a NHIEP sequence, or "knock-outs" (which can be conditional) that do not express

a functional NHIEP. Knock-out mice can be produced in several ways, one of which involves the use of mouse embryonic stem cells ("ES cells") lines that contain gene trap mutations in a murine homolog of at least one of the described NHIEPs. When the unique NHIEP sequences described in SEQ ID NOS:1-5 are "knocked-out" they provide a method of identifying phenotypic expression of the particular gene as well as a method of assigning function to previously unknown genes. In addition, animals in which the unique NHIEP sequences described in SEQ ID NOS:1-5 are "knocked-out" provide a unique source in which to elicit antibodies to homologous and orthologous proteins which would have been previously viewed by the immune system as "self" and therefore would have failed to elicit significant antibody responses.

Additionally, the unique NHIEP sequences described in SEQ ID NOS:1-5 are useful for the identification of protein coding sequence and mapping a unique gene to a particular chromosome. These sequences identify actual, biologically verified, and therefore relevant, exon splice junctions as opposed to those that may have been bioinformatically predicted from genomic sequence alone. The sequences of the present invention are also useful as additional DNA markers for restriction fragment length polymorphism (RFLP) analysis, and in forensic biology.

Further, the present invention also relates to processes for identifying compounds that modulate, *i.e.*, act as agonists or antagonists, of NHIEP expression and/or NHIEP activity that utilize purified preparations of the described NHIEPs and/or NHIEP product, or cells expressing the same. Such compounds can be used as therapeutic agents for the treatment of any of a wide variety of symptoms associated with biological disorders or imbalances.

4. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES

The Sequence Listing provides the sequences of the described NHIEP ORFs that encode the described NHIEP amino acid sequences. SEQ ID NO:5 describes a polynucleotide encoding a NHIEP ORF with regions of flanking sequence.

5. DETAILED DESCRIPTION OF THE INVENTION

The NHIEPs described for the first time herein are novel proteins that may be expressed in, *inter alia,* human cell lines, fetal brain, brain, pituitary, cerebellum, spinal cord, lymph node, lung, prostate, adrenal gland, skeletal muscle, esophagus, pericardium, hypothalamus, fetal kidney, tongue, 6-12 12 week embryos, and osteosarcoma cells.

The present invention encompasses the nucleotides presented in the Sequence Listing, host cells expressing such nucleotides, the expression products of such nucleotides, and: (a) nucleotides that encode mammalian homologs of the described genes, including the specifically described NHIEPs, and the NHIEP products; (b) nucleotides that encode one or more portions of the NHIEPs that correspond to functional domains, and the polypeptide products specified by such nucleotide sequences, including, but not limited to, the novel regions of any active domain(s); (c) isolated nucleotides that encode mutant versions, engineered or naturally occurring, of the described NHIEPs in which all or a part of at least one domain is deleted or altered, and the polypeptide products specified by such nucleotide sequences, including, but not limited to, soluble proteins and peptides in which all or a portion of the signal (or hydrophobic transmembrane) sequence is deleted; (d) nucleotides that encode chimeric fusion proteins containing all or a portion of a coding region of an NHIEP, or one of its domains (*e.g.*, a receptor or ligand binding domain, accessory protein/self-association domain, *etc.*) fused to another peptide

or polypeptide; or (e) therapeutic or diagnostic derivatives of the described polynucleotides such as oligonucleotides, antisense polynucleotides, ribozymes, dsRNA, or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing.

As discussed above, the present invention includes: (a) the human DNA sequences presented in the Sequence Listing (and vectors comprising the same) and additionally contemplates any nucleotide sequence encoding a contiguous NHIEP open reading frame (ORF) that hybridizes to a complement of a DNA sequence presented in the Sequence Listing under highly stringent conditions, *e.g.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., NY, at p. 2.10.3) and encodes a functionally equivalent expression product. Additionally contemplated are any nucleotide sequences that hybridize to the complement of a DNA sequence that encodes and expresses an amino acid sequence presented in the Sequence Listing under moderately stringent conditions, *e.g.*, washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, *supra*), yet still encodes a functionally equivalent NHIEP product. Functional equivalents of a NHIEP include naturally occurring NHIEPs present in other species and mutant NHIEPs whether naturally occurring or engineered (by site directed mutagenesis, gene shuffling, directed evolution as described in, for example, U.S. Patent No. 5,837,458). The invention also includes degenerate nucleic acid variants of the disclosed NHIEP polynucleotide sequences.

Additionally contemplated are polynucleotides encoding NHIEP ORFs, or their functional equivalents, encoded by polynucleotide sequences that are about 99, 95, 90, or about

85 percent similar or identical to corresponding regions of the nucleotide sequences of the Sequence Listing (as measured by BLAST sequence comparison analysis using, for example, the GCG sequence analysis package using standard default settings).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the described NHIEP gene nucleotide sequences. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances where the nucleic acid molecules are deoxyoligonucleotides ("DNA oligos"), such molecules are generally about 16 to about 100 bases long, or about 20 to about 80, or about 34 to about 45 bases long, or any variation or combination of sizes represented therein that incorporate a contiguous region of sequence first disclosed in the Sequence Listing. Such oligonucleotides can be used in conjunction with the polymerase chain reaction (PCR) to screen libraries, isolate clones, and prepare cloning and sequencing templates, *etc.*

Alternatively, such NHIEP oligonucleotides can be used as hybridization probes for screening libraries, and assessing gene expression patterns (particularly using a micro array or high-throughput "chip" format). Additionally, a series of the described NHIEP oligonucleotide sequences, or the complements thereof, can be used to represent all or a portion of the described NHIEP sequences. An oligonucleotide or polynucleotide sequence first disclosed in at least a portion of one or more of the sequences of SEQ ID NOS: 1-5 can be used as a hybridization probe in conjunction with a solid support matrix/substrate (resins, beads, membranes, plastics, polymers, metal or metallized substrates, crystalline or polycrystalline substrates, *etc.*). Of particular note are spatially addressable arrays (*i.e.*, gene chips, microtiter plates, *etc.*) of oligonucleotides and polynucleotides, or corresponding oligopeptides and polypeptides, wherein at least one of the

biopolymers present on the spatially addressable array comprises an oligonucleotide or polynucleotide sequence first disclosed in at least one of the sequences of SEQ ID NOS: 1-5, or an amino acid sequence encoded thereby. Methods for attaching biopolymers to, or synthesizing biopolymers on, solid support matrices, and conducting binding studies thereon are disclosed in, *inter alia*, U.S. Patent Nos. 5,700,637, 5,556,752, 5,744,305, 4,631,211, 5,445,934, 5,252,743, 4,713,326, 5,424,186, and 4,689,405 the disclosures of which are herein incorporated by reference in their entirety.

Addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-5 can be used to identify and characterize the temporal and tissue specific expression of a gene. These addressable arrays incorporate oligonucleotide sequences of sufficient length to confer the required specificity, yet be within the limitations of the production technology. The length of these probes is within a range of between about 8 to about 2000 nucleotides. Preferably the probes consist of 60 nucleotides and more preferably 25 nucleotides from the sequences first disclosed in SEQ ID NOS:1-5.

For example, a series of the described oligonucleotide sequences, or the complements thereof, can be used in chip format to represent all or a portion of the described sequences. The oligonucleotides, typically between about 16 to about 40 (or any whole number within the stated range) nucleotides in length can partially overlap each other and/or the sequence may be represented using oligonucleotides that do not overlap. Accordingly, the described polynucleotide sequences shall typically comprise at least about two or three distinct oligonucleotide sequences of at least about 8 nucleotides in length that are each first disclosed in the described Sequence Listing. Such oligonucleotide sequences can begin at any nucleotide present within a sequence in the Sequence Listing and proceed in either a sense (5'-to-3') orientation vis-a-vis the described sequence or in an antisense orientation.

Microarray-based analysis allows the discovery of broad patterns of genetic activity, providing new understanding of gene functions and generating novel and unexpected insight into transcriptional processes and biological mechanisms. The use of addressable arrays comprising sequences first disclosed in SEQ ID NOS:1-5 provides detailed information about transcriptional changes involved in a specific pathway, potentially leading to the identification of novel components or gene functions that manifest themselves as novel phenotypes.

Probes consisting of sequences first disclosed in SEQ ID NOS:1-5 can also be used in the identification, selection and validation of novel molecular targets for drug discovery. The use of these unique sequences permits the direct confirmation of drug targets and recognition of drug dependent changes in gene expression that are modulated through pathways distinct from the drugs intended target. These unique sequences therefore also have utility in defining and monitoring both drug action and toxicity.

As an example of utility, the sequences first disclosed in SEQ ID NOS:1-5 can be utilized in microarrays or other assay formats, to screen collections of genetic material from patients who have a particular medical condition. These investigations can also be carried out using the sequences first disclosed in SEQ ID NOS:1-5 *in silico* and by comparing previously collected genetic databases and the disclosed sequences using computer software known to those in the art.

Thus the sequences first disclosed in SEQ ID NOS:1-5 can be used to identify mutations associated with a particular disease and also as a diagnostic or prognostic assay.

Although the presently described sequences have been specifically described using nucleotide sequence, it should be appreciated that each of the sequences can uniquely be described using any of a wide variety of additional structural attributes, or combinations thereof. For example, a given sequence can be described by the net composition of the nucleotides present within a given region of the sequence in conjunction with the presence of one or more specific

oligonucleotide sequence(s) first disclosed in the SEQ ID NOS: 1-5. Alternatively, a restriction map specifying the relative positions of restriction endonuclease digestion sites, or various palindromic or other specific oligonucleotide sequences can be used to structurally describe a given sequence. Such restriction maps, which are typically generated by widely available computer programs (*e.g.*, the University of Wisconsin GCG sequence analysis package, SEQUENCHER 3.0, Gene Codes Corp., Ann Arbor, MI, *etc.*), can optionally be used in conjunction with one or more discrete nucleotide sequence(s) present in the sequence that can be described by the relative position of the sequence relative to one or more additional sequence(s) or one or more restriction sites present in the disclosed sequence.

For oligonucleotide probes, highly stringent conditions may refer, *e.g.*, to washing in 6x SSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid molecules may encode or act as NHIEP gene antisense molecules, useful, for example, in NHIEP gene regulation and/or as antisense primers in amplification reactions of NHIEP gene nucleic acid sequences. With respect to NHIEP gene regulation, such techniques can be used to regulate biological functions. Further, such sequences may be used as part of ribozyme and/or triple helix sequences that are also useful for NHIEP gene regulation.

Inhibitory antisense or double stranded oligonucleotides can additionally comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine,

2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide will comprise at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier *et al.*, 1987, Nucl. Acids Res. *15*:6625-6641). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue *et al.*, 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, 1987, FEBS Lett. *215*:327-330). Alternatively, double stranded RNA can be used to disrupt the expression and function of a targeted NHIEP.

Oligonucleotides of the invention can be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, *etc.*). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein *et al.* (1988, Nucl. Acids Res. *16*:3209), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. *85*:7448-7451), *etc.*

Low stringency conditions are well-known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual (and periodic updates thereof), Cold Harbor Press, NY; and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, NY.

Alternatively, suitably labeled NHIEP nucleotide probes can be used to screen a human genomic library using appropriately stringent conditions or by PCR. The identification and characterization of human genomic clones is helpful for identifying polymorphisms (including, but not limited to, nucleotide repeats, microsatellite alleles, single nucleotide polymorphisms, or coding single nucleotide polymorphisms), determining the genomic structure of a given locus/allele, and designing diagnostic tests. For example, sequences derived from regions adjacent to the intron/exon boundaries of the human gene can be used to design primers for use in amplification assays to detect mutations within the exons, introns, splice sites (*e.g.*, splice acceptor and/or donor sites), *etc.*, that can be used in diagnostics and pharmacogenomics.

For example, the present sequences can be used in restriction fragment length polymorphism (RFLP) analysis to identify specific individuals. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification (as generally described in U.S. Patent No. 5,272,057, incorporated herein by reference).

In addition, the sequences of the present invention can be used to provide polynucleotide reagents, *e.g.*, PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (*i.e.*, another DNA sequence that is unique to a particular individual). Actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments.

Further, a NHIEP gene homolog can be isolated from nucleic acid from an organism of interest by performing PCR using two degenerate or "wobble" oligonucleotide primer pools designed on the basis of amino acid sequences within the NHIEP products disclosed herein. The template for the reaction may be total RNA, mRNA, and/or cDNA obtained by reverse transcription of mRNA prepared from human or non-human cell lines or tissue known or suspected to express an allele of a NHIEP gene. The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequence of the desired NHIEP gene. The PCR fragment can then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment can be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment can be used to isolate genomic clones via the screening of a genomic library.

PCR technology can also be used to isolate full length cDNA sequences. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source (*i.e.*, one known, or suspected, to express a NHIEP gene). A reverse transcription (RT) reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a complementary primer. Thus, cDNA sequences upstream of the amplified fragment can be isolated. For a review of cloning strategies that can be used, see *e.g.*, Sambrook *et al.*, 1989, *supra*.

A cDNA encoding a mutant NHIEP sequence can be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying a mutant NHIEP allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal sequence. Using these two primers, the product is then amplified via PCR, optionally cloned into a suitable vector, and subjected to DNA sequence analysis through methods well-known to those of skill in the art. By comparing the DNA sequence of the mutant NHIEP allele to that of a corresponding normal NHIEP allele, the mutation(s) responsible for the loss or alteration of function of the mutant NHIEP gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual suspected of or known to carry a mutant NHIEP allele (*e.g.*, a person manifesting a NHIEP-

associated phenotype such as, for example, osteoporosis, obesity, high blood pressure, connective tissue disorders, infertility, *etc.*), or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant NHIEP allele. A normal NHIEP gene, or any suitable fragment thereof, can then be labeled and used as a probe to identify the corresponding mutant NHIEP allele in such libraries. Clones containing mutant NHIEP sequences can then be purified and subjected to sequence analysis according to methods well-known to those skilled in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant NHIEP allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue can be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against a normal NHIEP product, as described below. For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, NY.

Additionally, screening can be accomplished by screening with labeled NHIEP fusion proteins, such as, for example, alkaline phosphatase-NHIEP or NHIEP-alkaline phosphatase fusion proteins. In cases where a NHIEP mutation results in an expression product with altered function (*e.g.*, as a result of a missense or a frameshift mutation), polyclonal antibodies to NHIEP are likely to cross-react with a corresponding mutant NHIEP expression product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well-known in the art.

The invention also encompasses (a) DNA vectors that contain any of the foregoing NHIEP coding sequences and/or their complements (*i.e.*, antisense); (b) DNA expression vectors that contain any of the foregoing NHIEP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences (for example, baculovirus as described in U.S. Patent No. 5,869,336 herein incorporated by reference); (c) genetically engineered host cells that contain any of the foregoing NHIEP coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous NHIEP sequence under the control of an exogenously introduced regulatory element (*i.e.*, gene activation). As used herein, regulatory elements include, but are not limited to, inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include, but are not limited to, the cytomegalovirus (hCMV) immediate early gene, regulatable, viral elements (particularly retroviral LTR promoters), the early or late promoters of SV40 adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase (PGK), the promoters of acid phosphatase, and the promoters of the yeast α-mating factors.

The present invention also encompasses antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists and agonists of a NHIEP, as well as compounds or nucleotide constructs that inhibit expression of a NHIEP sequence (transcription factor inhibitors, antisense and ribozyme molecules, or open reading frame sequence or regulatory sequence replacement constructs), or promote the

expression of a NHIEP (*e.g.*, expression constructs in which NHIEP coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, *etc.*).

The NHIEPs or NHIEP peptides, NHIEP fusion proteins, NHIEP nucleotide sequences, antibodies, antagonists and agonists can be useful for the detection of mutant NHIEPs or inappropriately expressed NHIEPs for the diagnosis of disease. The NHIEP proteins or peptides, NHIEP fusion proteins, NHIEP nucleotide sequences, host cell expression systems, antibodies, antagonists, agonists and genetically engineered cells and animals can be used for screening for drugs (or high throughput screening of combinatorial libraries) effective in the treatment of the symptomatic or phenotypic manifestations of perturbing the normal function of NHIEP in the body. The use of engineered host cells and/or animals may offer an advantage in that such systems allow not only for the identification of compounds that bind to the endogenous receptor for an NHIEP, but can also identify compounds that trigger NHIEP-mediated activities or pathways.

Finally, the NHIEP products can be used as therapeutics. For example, soluble derivatives such as NHIEP peptides/domains corresponding to NHIEPs, NHIEP fusion protein products (especially NHIEP-Ig fusion proteins, *i.e.*, fusions of a NHIEP, or a domain of a NHIEP, to an IgFc), NHIEP antibodies and anti-idiotypic antibodies (including Fab fragments), antagonists or agonists (including compounds that modulate or act on downstream targets in a NHIEP-mediated pathway) can be used to directly treat diseases or disorders. For instance, the administration of an effective amount of soluble NHIEP, or a NHIEP-IgFc fusion protein or an anti-idiotypic antibody (or its Fab) that mimics the NHIEP could activate or effectively antagonize the endogenous NHIEP receptor. Nucleotide

constructs encoding such NHIEP products can be used to genetically engineer host cells to express such products *in vivo*; these genetically engineered cells function as "bioreactors" in the body delivering a continuous supply of a NHIEP, a NHIEP peptide, or a NHIEP fusion protein to the body. Nucleotide constructs encoding functional NHIEPs, mutant NHIEPs, as well as antisense and ribozyme molecules can also be used in "gene therapy" approaches for the modulation of NHIEP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.

Various aspects of the invention are described in greater detail in the subsections below.

5.1 THE NHIEP SEQUENCES

The cDNA sequences and the corresponding deduced amino acid sequences of the described NHIEPs are presented in the Sequence Listing. The NHIEP nucleotides were obtained from clustered genomic sequence, ESTs, and cDNAs from a brain cDNA library (Edge Biosystems, Gaithersburg, MD).

Two polymorphisms were identified during the sequencing of the NHIEPs including an A/G polymorphism at the nucleotide position represented by, for example, position 1889 of SEQ ID NO:1 (which can result in an asp or gly at corresponding amino acid (aa) position 630 of, for example, SEQ ID NO:2), and either the presence or absence of an extra GCA triplet at nucleotide position 2113 (which can result in the addition of an extra ala at aa position 705 of, for example, SEQ ID NO:2). The present invention contemplates sequences comprising any of the above polymorphisms, as well as any and all combinations and permutations of the above. The gene encoding the described NHIEPs is apparently encoded on human chromosome 14, and thus the described NHIEPs can be used to map the coding regions of

the human genome, and particularly human chromosome 14 (see GENBANK accession no. AL135747).

The described novel human polynucleotide sequences can be used, among other things, in the molecular mutagenesis/evolution of proteins that are at least partially encoded by the described novel sequences using, for example, polynucleotide shuffling or related methodologies. Such approaches are described in U.S. Patent Nos. 5,830,721 and 5,837,458 which are herein incorporated by reference in their entirety.

NHIEP gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, worms, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, birds, goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate NHIEP transgenic animals.

Any technique known in the art may be used to introduce a NHIEP transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Patent No. 4,873,191); retrovirus-mediated gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. Natl. Acad. Sci. USA *82*:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell *56*:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. *3*:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell *57*:717-723); *etc.* For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. *115*:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the NHIEP transgene in all their cells, as well as animals which carry the transgene in some, but not all their

cells, *i.e.*, mosaic animals or somatic cell transgenic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell-type by following, for example, the teaching of Lasko *et al.*, 1992, Proc. Natl. Acad. Sci. USA *89*:6232-6236. The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell-type of interest, and will be apparent to those of skill in the art.

When it is desired that a NHIEP transgene be integrated into the chromosomal site of the endogenous NHIEP gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous NHIEP gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous NHIEP gene (*i.e.*, "knockout" animals).

The transgene can also be selectively introduced into a particular cell-type, thus inactivating the endogenous NHIEP gene in only that cell-type, by following, for example, the teaching of Gu *et al.*, 1994, Science, *265*:103-106. The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell-type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant NHIEP gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be

assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of NHIEP gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the NHIEP transgene product.

The present invention provides for "knockin" animals. Knockin animals are those in which a gene that the animal does not naturally have in its genome, is inserted. For example, when a human gene is used to replace its murine ortholog in the mouse. Such knockin animals are useful for the *in vivo* study, testing and validation of, *intra alia*, human drug targets as well as for compounds that are directed at the same.

5.2 NHIEPs AND NHIEP POLYPEPTIDES

NHIEPs, NHIEP polypeptides, peptide fragments, mutated, truncated, or deleted forms of the NHIEPs, and/or NHIEP fusion proteins can be prepared for a variety of uses. These uses include, but are not limited to, therapeutic products, the generation of antibodies, as reagents in diagnostic assays, the identification of other cellular gene products related to a NHIEP, as reagents in assays for screening for compounds that can be used as pharmaceutical reagents useful in the therapeutic treatment of mental, biological, or medical disorders and diseases. Given the similarity information and expression data, the described NHIEPs can be targeted (by drugs, oligos, antibodies, etc,) in order to treat disease, or to therapeutically augment the efficacy of, for example, chemotherapeutic agents used in the treatment of cancer, arthritis, or as antiviral agents.

The Sequence Listing discloses the amino acid sequences encoded by the described NHIEP sequences. The NHIEPs display initiator methionines in DNA sequence contexts consistent with

translation initiation sites, and a hydrophobic region near the N-terminus that may serve as a signal sequence which indicates that the described NHIEPs can be secreted, membrane-associated, or cytoplasmic.

The NHIEP amino acid sequences of the invention include the amino acid sequence presented in the Sequence Listing as well as analogues and derivatives thereof. Further, corresponding NHIEP homologues from other species are encompassed by the invention. In fact, any NHIEP protein encoded by the NHIEP nucleotide sequences described above are within the scope of the invention as are any novel polynucleotide sequences encoding all or any novel portion of an amino acid sequence presented in the Sequence Listing. The degenerate nature of the genetic code is well-known, and, accordingly, each amino acid presented in the Sequence Listing, is generically representative of the well-known nucleic acid "triplet" codon, or in many cases codons, that can encode the amino acid. As such, as contemplated herein, the amino acid sequences presented in the Sequence Listing, when taken together with the genetic code (see, for example, Table 4-1 at page 109 of "Molecular Cell Biology", 1986, J. Darnell *et al.* eds., Scientific American Books, New York, NY, herein incorporated by reference) are generically representative of all the various permutations and combinations of nucleic acid sequences that can encode such amino acid sequences.

The invention also encompasses proteins that are functionally equivalent to the NHIEPs encoded by the presently described nucleotide sequences as judged by any of a number of criteria, including, but not limited to, the ability to bind and cleave a substrate of a NHIEP, or the ability to effect an identical or complementary downstream pathway, or a change in cellular metabolism (*e.g.*, proteolytic activity, ion flux, tyrosine phosphorylation, *etc.*). Such functionally equivalent

NHIEP proteins include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the NHIEP nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent expression product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

A variety of host-expression vector systems can be used to express the NHIEP nucleotide sequences of the invention. Where, as in the present instance, the NHIEP peptide or polypeptide is thought to be membrane protein, the hydrophobic regions of the protein can be excised and the resulting soluble peptide or polypeptide can be recovered from the culture media. Such expression systems also encompass engineered host cells that express a NHIEP, or functional equivalent, *in situ*. Purification or enrichment of a NHIEP from such expression systems can be accomplished using appropriate detergents and lipid micelles and methods well-known to those skilled in the art. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and functional characteristics of the NHIEP, but to assess biological activity, *e.g.*, in certain drug screening assays.

The expression systems that may be used for purposes of the invention include, but are not limited to, microorganisms

such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing NHIEP nucleotide sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing NHIEP nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing NHIEP nucleotide sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing NHIEP nucleotide sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing NHIEP nucleotide sequences and promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the NHIEP product being expressed. For example, when a large quantity of such a protein is to be produced for the generation of pharmaceutical compositions of or containing NHIEP, or for raising antibodies to a NHIEP, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO J. *2*:1791), in which a NHIEP coding sequence may be ligated individually into the vector in frame with the *lacZ* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. *13*:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. *264*:5503-5509); and the like. pGEX vectors (Pharmacia or American Type Culture Collection) can also be used to express

foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target expression product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign polynucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. A NHIEP coding sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of NHIEP coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted sequence is expressed (*e.g.*, see Smith *et al.*, 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the NHIEP nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing

a NHIEP product in infected hosts (*e.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA *81*:3655-3659). Specific initiation signals may also be required for efficient translation of inserted NHIEP nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire NHIEP gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of a NHIEP coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.* (See Bitter *et al.*, 1987, Methods in Enzymol. *153*:516-544).

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the expression product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and expression products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and

phosphorylation of the expression product may be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, human cell lines.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the NHIEP sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the NHIEP product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the NHIEP product.

A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell *11*:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, 1962, Proc. Natl. Acad. Sci. USA *48*:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell *22*:817) genes, which can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which

confers resistance to methotrexate (Wigler *et al.*, 1980, Proc. Natl. Acad. Sci. USA 77:3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA *78*:1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, 1981, Proc. Natl. Acad. Sci. USA *78*:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. *150*:1); and hygro, which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene *30*:147).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, *et al.*, 1991, Proc. Natl. Acad. Sci. USA *88*:8972-8976). In this system, the sequence of interest is subcloned into a vaccinia recombination plasmid such that the sequence's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+}·nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Also encompassed by the present invention are fusion proteins that direct the NHIEP to a target organ and/or facilitate transport across the membrane into the cytosol. Conjugation of NHIEPs to antibody molecules or their Fab fragments could be used to target cells bearing a particular epitope. Attaching the appropriate signal sequence to the NHIEP would also transport the NHIEP to the desired location within the cell. Alternatively targeting of NHIEP or its nucleic acid sequence might be achieved using liposome or lipid complex based delivery systems. Such technologies are described in "Liposomes:A Practical Approach", New, R.R.C.,

ed., Oxford University Press, New York and in U.S. Patent Nos. 4,594,595, 5,459,127, 5,948,767 and 6,110,490 and their respective disclosures which are herein incorporated by reference in their entirety. Additionally embodied are novel protein constructs engineered in such a way that they facilitate transport of the NHIEP to the target site or desired organ, where they cross the cell membrane and/or the nucleus where the NHIEP can exert its functional activity. This goal may be achieved by coupling of the NHIEP to a cytokine or other ligand that provides targeting specificity, and/or to a protein transducing domain (see generally U.S. applications Ser. Nos. 60/111,701 and 60/056,713, both of which are herein incorporated by reference, for examples of such transducing sequences) to facilitate passage across cellular membranes and can optionally be engineered to include nuclear localization.

5.3 ANTIBODIES TO NHIEP PRODUCTS

Antibodies that specifically recognize one or more epitopes of a NHIEP, or epitopes of conserved variants of a NHIEP, or peptide fragments of a NHIEP are also encompassed by the invention. Such antibodies include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of NHIEP in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of NHIEP. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes for the evaluation of the effect of test compounds on expression and/or

activity of a NHIEP expression product. Additionally, such antibodies can be used in conjunction gene therapy to, for example, evaluate the normal and/or engineered NHIEP-expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal NHIEP activity. Thus, such antibodies may, therefore, be utilized as part of treatment methods.

For the production of antibodies, various host animals may be immunized by injection with a NHIEP, an NHIEP peptide (*e.g.*, one corresponding to a functional domain of an NHIEP), truncated NHIEP polypeptides (NHIEP in which one or more domains have been deleted), functional equivalents of the NHIEP or mutated variant of the NHIEP. Such host animals may include, but are not limited to pigs, rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including, but not limited to, Freund's adjuvant (complete and incomplete), mineral salts such as aluminum hydroxide or aluminum phosphate, chitosan, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Alternatively, the immune response could be enhanced by combination and or coupling with molecules such as keyhole limpet hemocyanin, tetanus toxoid, diphtheria toxoid, ovalbumin, cholera toxin or fragments thereof. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and

Milstein, (1975, Nature *256*:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, Immunology Today *4*:72; Cole *et al.*, 1983, Proc. Natl. Acad. Sci. USA *80*:2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, Proc. Natl. Acad. Sci. USA, *81*:6851-6855; Neuberger *et al.*, 1984, Nature, *312*:604-608; Takeda *et al.*, 1985, Nature, *314*:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Such technologies are described in U.S. Patent Nos. 6,075,181 and 5,877,397 and their respective disclosures which are herein incorporated by reference in their entirety. Also encompassed by the present invention is the use of fully humanized monoclonal antibodies as described in US Patent No. 6,150,584 and respective disclosures which are herein incorporated by reference in their entirety.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science *242*:423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA *85*:5879-5883; and Ward *et al.*, 1989, Nature *341*:544-

546) can be adapted to produce single chain antibodies against NHIEP expression products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, Science, *246*:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to a NHIEP can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" a given NHIEP, using techniques well-known to those skilled in the art. (See, *e.g.*, Greenspan & Bona, 1993, FASEB J *7(5)*:437-444; and Nissinoff, 1991, J. Immunol. *147(8)*:2429-2438). For example antibodies which bind to a NHIEP domain and competitively inhibit the binding of NHIEP to its cognate receptor can be used to generate anti-idiotypes that "mimic" the NHIEP and, therefore, bind and activate or neutralize a receptor. Such anti-idiotypic antibodies or Fab fragments of such anti-idiotypes can be used in therapeutic regimens involving a NHIEP-mediated pathway.

Additionally given the high degree of relatedness of mammalian NHIEPs, the presently described knock-out mice (having never seen NHIEP, and thus never been tolerized to NHIEP) have a unique utility, as they can be advantageously applied to the generation of antibodies against the disclosed mammalian NHIEP (*i.e.*, NHIEP will be immunogenic in NHIEP knock-out animals).

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims. All cited publications, patents, and patent applications are herein incorporated by reference in their entirety.